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TECHNICAL MANUSCRIPT 20

THE GROWTH OF ASIBI STRAIN  
YELLOW FEVER VIRUS  
IN TISSUE CULTURES

II. MODIFICATION OF  
VIRUS AND CELLS

NOVEMBER 1962

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Frank M. Hardy

U. S. ARMY MEDICAL UNIT

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### ABSTRACT

Passage of the parent Asibi strain yellow fever virus in HeLa cell culture through six consecutive passages produced a variant (H-6) that, unlike P virus: (a) induced cytopathic effect in HeLa and chick fibroblast cell cultures; (b) became more virulent for mice, and (c) lost its "viscerotropic" character for the monkey.

Chronic infection of HeLa cells with P virus was established for a two-month period. The virus obtained from this culture showed characteristics of the P and H-6 and was designated parent chronic (Pc).

The Pc virus strain was undetectable from supernatant fluid two months post-infection. Cloned populations of the surviving cells showed evidence of the modification induced by the Pc virus in terms of susceptibility (sensitivity and capacity).

### ACKNOWLEDGMENTS

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## I. INTRODUCTION

In studies with the intact animal, Theiler<sup>1</sup> reported modification of the Asibi strain as a result of serial mouse brain passage. The altered progeny differed from its parent (P) according to two criteria: (a) mice infected with lethal doses of the former succumbed more rapidly with equivalent virus concentrations; and (b) monkeys showed the "neurotropic" rather than the "viscerotropic" syndrome. The viscerotropic marker was shown by Hallauer<sup>2</sup> to decline when Asibi strain virus was passaged in KB cells.

Some of the early investigators<sup>3</sup> reported that chick embryo tissue cultures supported the growth of a French (neurotropic) strain, while others<sup>4</sup> showed this tissue culture to be insusceptible to the viscerotropic (Asibi) strain. Although the primary passage of Asibi strain in tissue cultures of chick embryo proved to be unsuccessful, the virus progeny of Asibi strain following 18 passages in minced mouse embryo culture was found to multiply in chick embryo tissue culture. A comparable change in viral properties after passage can be further illustrated by the 17D strain (an Asibi-derived strain), which was altered by continued passage through several hosts including chick embryo tissue culture. This 17D variant proved to be useful as an attenuated vaccine for man.

The purpose of this paper is to report on the changes in certain virus and cellular properties following the passage of Asibi strain virus in Hela cell cultures.

## II. METHODS

Virus strain, cell line, virus titration, and wash treatment procedures have been previously described.<sup>5</sup>

Plaque Assay - a modification of the monolayer plaque method described for the production of plaques with Venezuelan equine encephalomyelitis virus<sup>6</sup> was employed. Cultures overlaid with the nutrient agar solution were incubated up to six days at a temperature of 35°C.

### III. RESULTS

#### A. PASSAGE THROUGH HeLa CELLS

In an effort to induce modification of the P Asibi strain, virus was serially passaged in HeLa cultures. Virus contained in a one-milliliter volume of a  $10^{-2}$  dilution was inoculated into T-60 Earle flasks containing a monolayer of HeLa cells. Each culture was subjected to two wash treatments, one immediately before and the other one hour following inoculation. The latter wash treatment was adopted to effect a further dilution of the unadsorbed virus and to eliminate interference as described for the early phases of the infectious cycle in this system. Prior to harvest the only addition to an infected culture was bicarbonate and this only in concentrations sufficient to maintain a pH level of approximately 7.2. Throughout this series infected cultures were incubated at  $37^{\circ}\text{C}$  and harvested at arbitrary intervals; each interval was not less than 96 hours.

The results presented in Figure 1 show that a cytopathic effect (CPE) occurred starting at the third passage and became regular and predictable by the sixth passage. The CPE occurring at the sixth passage was characterized by progressive retraction of the cytoplasm with granulation and the appearance of cellular debris. Since HeLa cells infected with the P Asibi strain showed no evidence of CPE, the virus progeny of the sixth HeLa passage was considered to represent a variant population (H-6).

#### B. COMPARISON OF VARIOUS PROPERTIES OF P AND H-6 GROWTH CURVE

A growth-curve experiment was performed with P and H-6 in HeLa cultures for the purpose of further characterization of these strains. Paired HeLa cell cultures were inoculated with  $10^4$  MICLD<sub>50</sub> of each strain in one-milliliter volume. After the cultures were subjected to post-inoculation wash treatment and sampled for virus content, one T-60 Earle flask from each pair was placed at  $35^{\circ}\text{C}$ , the other at  $37^{\circ}\text{C}$ , and incubated for a six-day period. A 0.5-milliliter volume was removed daily and titrated in mice. The results of this experiment are shown in Figure 2 and indicate that, although exponential phases of virus growth in both strains were more or less parallel, H-6 attained approximately one log higher peak titer than P. Inasmuch as peak titers obtained with P virus through the course of many experiments have fluctuated between  $10^6$  and  $10^7$  and those of H-6 ranged between  $10^7$  and  $10^8$ , this difference of one log was considered significant.

#### C. VIRULENCE FOR MICE

The H-6 strain also appeared to be more virulent for mice. With equivalent doses, mice began to die two to three days earlier with H-6 than with the P strain of virus.

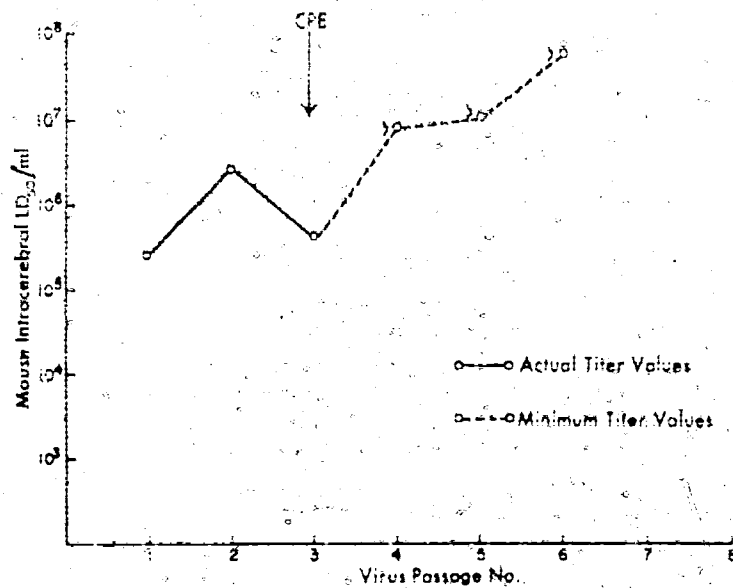


Figure 1. Rapid Passage of Yellow Fever Virus in HeLa Cell Cultures.

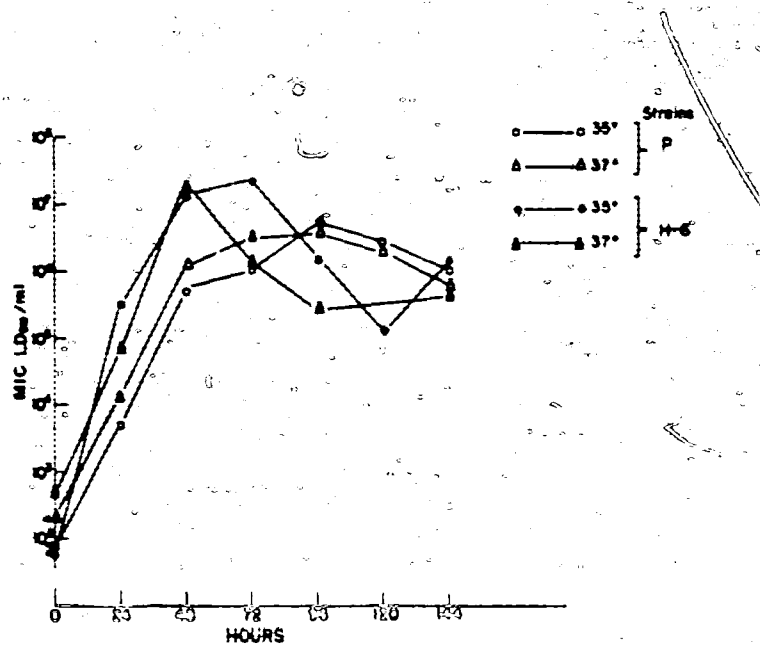


Figure 2. Growth of P and H-6 Strains of Yellow Fever Virus in HeLa Cultures at 35° and 37°C.

#### D. VIRULENCE FOR MONKEYS

From experiments performed with monkeys, it was evident that H-6 differed from P virus in that monkeys infected with the HeLa strain did not exhibit a vicerotropic disease syndrome as observed in those infected with P virus.

#### E. PLAQUE FORMATION

A comparison of the two strains with respect to their plaque-forming ability in both HeLa and chick fibroblasts was next investigated. Results of these experiments showed that only the H-6 strain had the ability to form plaques when either HeLa or chick fibroblast were employed, and that the plaques appeared after the fifth day of incubation. In parallel titrations of the H-6 strain, two logs less virus were detectable by the plaque method than in mice that were inoculated by the intracerebral route.

#### F. ANTIGENIC IDENTITY OF P AND H-6

Neutralization tests were performed to establish virus identity and specificity employing mouse brain seed of P virus and tissue-culture-grown H-6 virus. In this experiment the virus contained in both the inoculum and harvest were titrated against Asibi strain immune sera obtained from two sources: (a) a guinea pig immune serum from animals immunized with mouse brain seed, and (b) monkey antiserum\* immunized with a monkey plasma virus seed. Results of these neutralization tests with normal serum controls showed that H-6 and the Asibi strain seed virus were antigenically equivalent; that is, antisera to both strains were equivalent in their neutralization indexes. Table I summarizes the differences between the H-6 and P strains of yellow fever virus.

TABLE I. CHARACTERISTICS OF TWO STRAINS OF YELLOW FEVER VIRUS

Characteristics	Parent (P) Asibi strain	Variant (H-6) HeLa-Cell-Derived (6th passage)
Peak titer from HeLa cell culture in MICLD <sub>50</sub> /ml	10 <sup>7</sup>	10 <sup>8</sup>
Time of cytopathogenic effect		
(1) in HeLa cells		4 days
(2) in chick fibroblasts		4 days
Day of initial mortality in mice	8 to 9th day	6 to 7th day
Monkey virulence	Lethal	Avirulent

\* Prepared by Mr. E.C. Corrigan.

## G. CHRONIC INFECTION IN HeLa CELLS

In an effort to establish a chronic infection of Asibi strain virus in HeLa cells, a culture was inoculated with  $10^4$  MICLD<sub>50</sub>. The culture was subjected to a wash treatment one hour post-inoculation and then incubated for six days. After the sixth day, it was further wash-treated from the twelfth to the sixtieth day at intervals of three or six days. Results of this experiment (Figure 3) suggest that a continuous production and release of yellow fever virus occurred during the two-month period. Titers regularly ranged between  $10^5$  and  $10^6$  MICLD<sub>50</sub>/ml from the ninth to the sixtieth day, although the dilution of the virus resulting from the medium replacement represented a greater dilution of virus than could have possibly been in the inoculum.

Virus harvested from this culture was designated parent chronic (Pc). A CPE was observed in this culture at the twelfth day of incubation, but after a change of medium on the fifteenth day, morphological characteristics of the surviving cells remained indistinguishable from uninfected control cultures.

Greater bicarbonate concentration was necessary to maintain pH levels above 7.2 in the Pc cultures than in uninfected control cultures. The increased acidity in infected cultures has been associated with viruses other than the group B arthropod-borne viruses.<sup>7</sup>

The Pc virus recovered from the supernatant fluid samples of this culture after 1½ months resembled H-6 more than P virus. HeLa cells and chick fibroblasts showed progressive CPE when inoculated with this virus, although the pattern of mouse deaths observed following inoculation was indistinguishable from that previously experienced with P virus. Thus, it was concluded that the modification of Asibi virus resulting from continuous association with HeLa cells for a two-month period either had not progressed to the degree shown by the H-6 variant and/or resulted in a different variant population.

The surviving cells of the Pc virus culture were carried for an additional month as a stock culture (medium changes followed by subcultures when cell sheets became confluent). These cells were cloned (through one transfer) according to the method followed previously.<sup>5</sup> From these cell populations only morphologically D-type clones were obtained.

Efforts to detect minimal virus concentrations from supernatant fluids were unsuccessful by either intracerebral inoculation of mice or plaque assay methods. It was assumed, therefore, that the long-term cell-virus association had not only altered the virus populations but had also altered the cell population, so that only morphologically D-type clones were recovered.

Inasmuch as Pc virus could not be detected from the surviving cell cultures, it was of interest to determine whether new infectious cycles could be supported by these D-type remnant populations that would also show cell or virus modification. Three cultures were inoculated with  $10^4$  MICLD<sub>50</sub> concentrations of virus, two with P and the other with H-6. Cultures were incubated for six days at 37 °C. The titration endpoints of daily samples are shown in Figure 4. The initial phase of virus growth, i.e., from zero

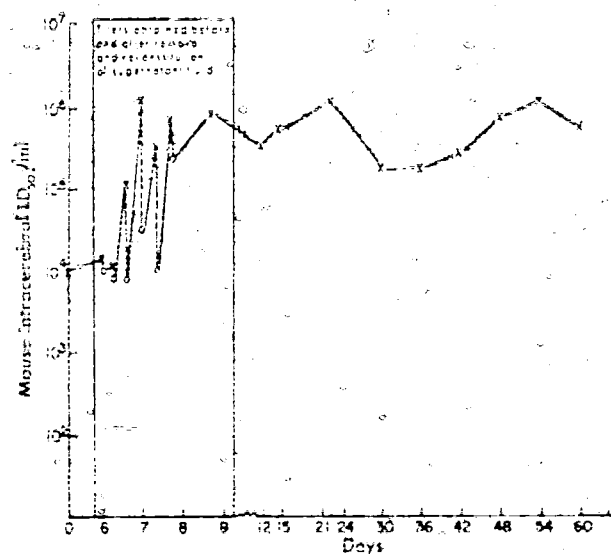


Figure 3. Continuous Liberation of Yellow Fever Virus from a Single Infection of a HeLa Culture. (Chronic Infection).

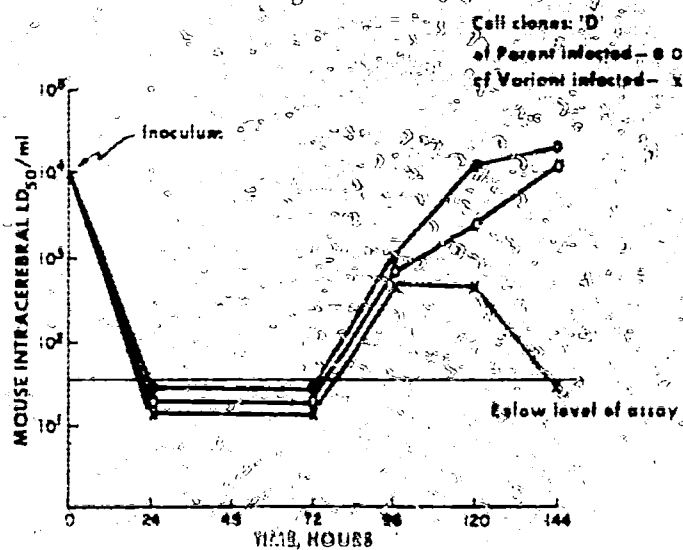


Figure 4. Challenge with Yellow Fever Virus, A&ibi Strain, in Surviving HeLa Cells Initially Infected with Parent or Variant Virus.

through 96 hours, showed no evidence of modification, either of virus (B or H-6) or of cells ("normal" or previously infected). In contrast, differences during the late phase of virus growth were manifest: (a) the culmination of the exponential growth of P virus in D-type cells surviving Pc virus infection attained a peak titer of  $10^4$ , a titer of two logs less than was obtained in D clones having no prior experience with this virus, (b) peak virus titers of H-6 virus were approximately  $10^{2.2}$ /ml, terminating in virus concentrations below the level of assay. It was apparent, therefore, that the resulting continuous virus-cell interaction had modified both virus and cell populations. Whether re-infected with the P strain or primarily infected with H-6, the surviving cells of the morphological D-type clones showed no effect on the early phase of virus infection, but there was a modification of the capacity of these cells to support virus growth. The effect on capacity was shown to be greater with H-6 than with the P strain virus in D-type clones of surviving cells. Although the D-type clones tested may be composed of mixed populations, since they resulted from only one transfer, it should be noted that inoculation of HeLa cells with  $10^4$  MICLD<sub>50</sub> of virus, regardless of clone, always produced a titer higher than  $10^5$  MICLD<sub>50</sub>/ml and that the D-type cells represented the vast majority.

#### IV. DISCUSSION

Tests performed with the P Asibi strain virus indicated that no change had occurred in the properties associated previously with this virus following a single passage in HeLa cell cultures. In contrast, virus obtained either from long-term virus-HeLa cell association, i.e., chronic infection for two months (Pc), or following six serial passages in HeLa cells (H-6), resulted in virus modification. No distinction can be made with either of these variant virus populations as to whether they were spontaneous mutants or that they represent the emergence of a subpopulation present in the original population of P virus. The Pc virus, unlike P, induced cytopathic changes when transferred to "normal" HeLa and chick fibroblast monolayer cultures. Even more marked were the modifications exhibited by the H-6 virus when compared with P. Mice infected with the H-6 virus died earlier than those infected with P virus. Monkeys exposed to H-6 survived; others, similarly infected with P virus, succumbed. Plaques were obtained in chick fibroblast cultures with H-6 but not with P. Apparently, H-6 represented a different or even greater modification of the initial virus population than did Pc. Although the times of Pc and H-6 virus-cell associations were comparable, the HeLa cell populations during the infectious cycles of Pc were continually undergoing modification, while the precursors of H-6 virus were exposed to an unmodified cellular environment, i.e., equivalent to that of P virus. The surviving HeLa population producing Pc did not undergo marked cytopathic alteration compared with those infected with H-6 virus. Additional modifications evident in the HeLa cell population that produced Pc indicated that only the capacity to produce high virus concentration and not the sensitivity to infection had been modified. Thus, D-type clones of the Pc culture when re-infected with P virus showed an initial infection cycle similar to "normal" D-type clones but terminated with considerably less virus produced. When H-6 virus was employed with the D-type (Pc remnant) clones, an even greater decline in capacity was detected. Thus, although the D-type clones isolated from parent HeLa cells and chronically infected HeLa were morphologically indistinguishable, they differed in susceptibility to P and H-6. During the modification of the HeLa cell population that resulted from Pc multiplication, a population shift in HeLa cells was observed to a new D-type clone.



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